

HIV-1 Induces Cytotoxic T Lymphocytes in the Cervix of Infected Women

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Summary

Although T lymphocytes are present in the genital mucosa, their function in sexually transmitted diseases is unproven. To determine if cervical T cells mediate HIV-specific cytolysis, mononuclear cells in cytobrush specimens from HIV-1-infected women were stimulated in vitro with antigen. Resultant cell lines lysed autologous targets expressing HIV-1 proteins in 12/19 (63%) subjects, and these responses were detected intermittently on repeated visits. All 8 subjects with blood CD4⁺ counts ≥ 500 cells/ μ l had HIV-1-specific cervical CTL, whereas only 4/11 with counts < 500 cells/ μ l had detectable responses ($P = 0.008$). Class II MHC-restricted CD4⁺ CTL clones lysed targets expressing Env gp41 or infected with HIV-1. Class I MHC-restricted CD8⁺ clones recognized HIV-1 Gag- or Pol-expressing targets, and the epitopes were mapped to within 9–20 amino acids. Comparisons of intra-individual cervical and blood CTL specificities indicate that epitopes recognized by CTL in the cervix were commonly recognized in the blood. These studies provide the first definitive evidence for an MHC-restricted effector function in human cervical lymphocytes.

More than 80% of HIV-1 transmissions occur through heterosexual intercourse, and the risk for acquiring infection by this route is at least twofold greater in women than men (1). The presence of HIV-specific effector immune responses at the site of transmission, the cervicovaginal area, may be critical in women for the initial control of infection and in the selection of strains which disseminate systemically. Local antibodies, particularly IgA, are the primary effectors classically responsible for the elimination of mucosal pathogens (2, 3). Although specific IgA antibodies are induced in HIV-1 infection, their presence in cervicovaginal fluid is neither correlated with reduced HIV-1 shedding nor protection from vertical transmission in pregnant women (4) and some investigations suggest that IgA may invoke antibody-mediated enhancement of infection (5, 6). Alternatively, mucosal T cells capable of both cytolytic activity and cytokine production may actively participate in the early defense against HIV-1 infection, as has been suggested from recent findings in the macaque model of SIV infection (7).

Intraepithelial and submucosal lymphocytes are present in the cervix and vagina of healthy adult women (8–10), and circumstantial evidence suggests that these cells participate in the immune response against foreign antigens. For example, in association with human papilloma virus infection (11), tobacco smoking (12), and dysplasia (13, 14), variations in the phenotypes of cervicovaginal T cell populations have been reported. Moreover, CD8⁺ T cells are

recruited to the squamocolumnar cervical junction in cervical neoplasia (15). Recently, Olaitan et al. (16) found significant alterations in the proportion of T lymphocytes, Langerhans' and plasma cells in the cervix of HIV-infected women. Thus far, however, there is no evidence that mucosal lymphocytes have an effector function, particularly cytolytic, in HIV infection.

The purpose of this investigation was to determine the presence and function of HIV-specific T cell responses in the cervix of HIV-infected women. Unexpectedly, we found that although CD3⁺ T cells comprise a small proportion ($< 5\%$) of the cells obtained from a cervical cytobrush or biopsy, selected populations of both CD4⁺ and CD8⁺ cells from these specimens were capable of HIV-specific cytolytic activity upon in vitro antigen-specific stimulation. We report the kinetics of responses in 19 HIV-1-infected women, the gene products recognized by the CTL, and the epitope specificity and MHC restriction patterns at the clonal level. These findings are the first to establish a functional role for intraepithelial lymphocytes in human cervical infection and indicate that the female genital mucosal system is armed not only with antibody but also cellular effector immune responses.

Materials and Methods

Study Population. 23 females with HIV-1 infection, documented serologically by both HIV-1 EIA and Western blot, and 5

HIV-1 seronegative female controls were enrolled in a longitudinal study to examine HIV-1-specific immunological and virological responses in the mucosal tissues. The University of Washington Human Subject Review Board approved all aspects of the investigation, and written consent was obtained from the volunteers before initiation of the study. The study entailed monthly visits by the volunteers to either the UW AIDS Vaccine Evaluation Unit or the Northwest Family Center. When appointments were missed, volunteers were rescheduled within 1–4 wk. At each visit, a clinician performed a clinical evaluation, pelvic examination and venipuncture. Peripheral blood T cell subset counts were measured by standard flow cytometric analysis in the University of Washington Hematopathology Laboratory. Class I and class II HLA serological typing was performed on PBMC at the Fred Hutchinson Cancer Research Center Clinical Immunogenetics Laboratory.

Collection and Processing of Cervical Specimens. Cervical specimens were collected by cytobrush and biopsy. No cervical specimens were taken from menstruating patients, if blood was visible in the cervical area, or if the epithelium appeared disrupted. The cytobrush was inserted just within the cervical os and rotated one 360° turn. Any specimen with visible blood was discarded. Immediately after sampling, the cytobrush was placed in a 15-ml conical tube containing 3 ml of RPMI 1640 with 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B (Biowhittaker, Walkersville, MD), and placed on ice. The specimen was transported on ice to the laboratory and processed within 3 h of collection. The cytobrush was gently rotated several times in the transport media and then discarded. The cell suspension was centrifuged (330 g, 10 min), and the pellet was resuspended in RPMI. Mononuclear cells were isolated by Ficoll-Hypaque density centrifugation, washed twice, and filtered through sterile cotton gauze to remove the majority of epithelial cells. After an additional wash by centrifugation, the mononuclear cells were resuspended in RPMI containing 100 U/ml penicillin, 100 µg/ml streptomycin, 10% heat-inactivated human AB serum (Biocell, Palo Alto, CA), and 50 µM β2-mercaptoethanol (Kodak, Rochester, NY), subsequently referred as R-10 HS. Samples containing red blood cells by light microscopy were discarded.

On selected volunteers, tissue biopsies of ~4 mm were obtained with forceps from the transitional zone of the cervix. After repeated washing to remove any contaminating blood, the cervical biopsies were gently pushed with a pestle through a 140-µm screen to obtain a single cell suspension, and then washed by centrifugation. Mononuclear cells were isolated by Ficoll-Hypaque density centrifugation, filtered through sterile cotton gauze, washed and resuspended in R-10 HS.

Flow Cytometry Analysis. The following mouse anti-human monoclonal antibodies were used to characterize subpopulations of mucosal lymphocytes: anti-CD3 FITC/CD16 PE, anti-CD4 FITC/CD8 PE, anti-CD14 FITC/CD45 PE, anti-CD19 FITC, and anti-TCR-αβ FITC/TCR-γδ PE (Becton-Dickinson, San Jose, CA), and FITC-conjugated anti-mucosal lymphocyte antigen (MLA-1) (Dako Corp., Carpinteria, CA). In brief, 10⁵ cells were incubated with the mAb for 30 min at 4°C, washed with PBS by centrifugation, fixed with 2% paraformaldehyde in PBS and analyzed with a FACScan® flow cytometer (Becton Dickinson). Samples were gated using the Consort-30 software (Becton Dickinson), and the appropriate isotype IgG controls were used to define background staining limits and quadrant markers. Scatter gates were first chosen to analyze the lymphocyte fraction and then expanded to include cells with greater forward and orthogonal scatter. Cell phenotypes were assessed by bivariate plots of FL-1 and FL-2 in logarithmic mode.

Recombinant Vaccinia Viruses. Recombinant vaccinia vectors (rVV)¹ were used to express HIV-1 or control gene products in target cells or monocyte stimulator cells. These included rVV encoding the full-length HIV-1_{LAI} envelope gp160 (vPE-16) (17), serial deletions of the HIV-1_{LAI} gp160 (vPE-17, vPE-18, vPE8, vPE21, vPE-22) (18), HIV-1_{LAI} *pol* (vRT) (19), and the control vector encoding lacZ (vSC-8) (20), which were kindly provided by B. Moss (NIH, Bethesda, MD). The rVV containing HIV-1_{LAI} *gag* (vDK-1) was provided by D. Kuritzkes (21), through the NIH AIDS Reagent Repository. The vaccinia vector encoding the three HIV-1_{LAI} genes *env*, *gag*, and *pol* (vv-EGP), was kindly provided by D. Panicali (Therion Inc., Cambridge, MA), and the rVV containing HIV-1 *gag* p24 (vP1287) and *gag* p17 (vP1289) by J. Tartaglia (Virogenetics, Inc., Troy, NY).

Synthetic HIV-1 Peptides. Synthetic peptides, up to 20 amino acids (aa) in length, corresponding to HIV-1 gene products were provided by Dr. M. C. Walker (NIAID, Bethesda, MD) through the NIH AIDS Reagent Repository, Dr. B. Walker (Massachusetts General Hospital, Boston, MA), and Dr. D. Lewinsohn (Fred Hutchinson Cancer Research Center, Seattle, WA). Peptides were reconstituted at a concentration of 1 mg/ml in 10% DMSO (Sigma Chemical Co., St. Louis, MO) and sterile H₂O, and were used at a final concentration of 10 µg/ml to pulse target cells for CTL assays.

In Vitro Stimulation of Effector CTL. Fresh autologous PBMC in RPMI (15 million in 3 ml) were allowed to adhere in a 6-well tissue culture plate at 37°C and 5% CO₂, and the nonadherent cells were removed after 2 h with repeated washes. The adherent monocytes were infected with vv-EGP (multiplicity of infection of 10) for 14 h at 37°C. The infected monocytes were UV-irradiated for 10 min, washed twice to remove any remaining nonadherent cells and free vaccinia virus, harvested gently with a cell scraper, gamma-irradiated (3,000 rad) and used as stimulator cells.

Freshly isolated mucosal mononuclear cells or PBMC were plated by limiting dilution in 96-well round-bottomed microtiter plates. Irradiated monocytes (1,000 cells) infected with vv-EGP and irradiated autologous PBMC (50,000 cells) feeder cells were resuspended in R-10 HS with 50 U human recombinant IL-2 (rIL-2) (Chiron, Emeryville, CA) and added in a volume of 100 µl to each microwell. The cultures were incubated at 37°C in 5% CO₂, and the wells were provided fresh media and rIL-2 twice weekly. Cells demonstrating growth after 2–4 wk were restimulated with 30 ng/ml OKT3 (Ortho Diagnostic System, Raritan, NJ), fresh irradiated feeder cells, and expanded into 24-well tissue culture plates (Costar, Cambridge, MA).

Preparation of Target Cells. Epstein-Barr virus transformed B lymphoblastoid cell lines (B-LCL) were established from peripheral blood mononuclear cells (PBMC) isolated from each subject and maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin 100 U/ml, and streptomycin 100 µg/ml (subsequently referred as R-10). Partially mismatched B-LCL from donors RML and SCHU were kindly provided by R. Sili-ciano (Johns Hopkins University, Baltimore, MD). Autologous or allogeneic B-LCL were infected with rVV at a multiplicity of infection of 1 for 16 h at 37°C, washed twice with R-10, and resuspended in 2 ml of R-10 before the CTL assay. To test effector lysis of target cells infected with HIV-1, B-LCL were transduced with a retroviral vector (LST4SN) containing the human CD4

¹Abbreviations used in this paper: aa, amino acids; rVV, recombinant vaccinia vectors.

Table 1. Cytolytic Responses of T Cells Isolated from Cervical Cytobrush Specimens in HIV-1-infected Women

Study Population			Specimens	HIV-1-Specific Lysis*			NK Lysis*	
Volunteer no.	CD4 count	Clinical course	No. evaluable/ no. collected	No. CTL+/ no. evaluable	HIV-1 Env	HIV-1 Gag	HIV-1 Pol	No. lysis+/ no. evaluable
	<i>cells/μl</i>							
704	673	oral/vag yeast	3/10	1/3	–	+	+‡	1/3
775	569	vag yeast	1/3	1/1	+	–	–	ND
709	1377	diarrhea, resolved	5/10	2/5	+	+	+	1/5
710	722	asymptomatic	10/10	6/10	+‡	+‡	–	1/10
909	969	vag yeast	1/2	1/1	–	+‡	–	0/1
742	500	asymptomatic	4/7	1/4	+	–	–	1/4
739	511	asymptomatic	2/3	1/2	+‡	–	+‡	0/2
741	563	asymptomatic	8/11	4/8	–	+‡	–	4/8
738	339	asymptomatic	8/8	4/8	+‡	+	+‡	2/8
743	83	vag yeast	2/7	0/2	–	–	–	1/2
747	204	yeast, trichomonas	2/3	1/2	+	–	–	1/2
770	178	diarrhea, resolved	1/5	0/1	–	–	–	0/1
711	415	dysplasia, vag yeast	2/7	0/2	–	–	–	0/2
776	230	deceased	1/2	0/1	–	–	–	0/1
824	273	cervical dysplasia	2/3	0/2	–	–	–	0/2
840	49	vag yeast	1/2	0/1	–	–	–	0/1
888	471	asymptomatic	2/3	0/2	–	–	–	0/2
737	356	vag yeast	3/6	1/3	+	–	–	3/3
708	450	asymptomatic	6/6	2/6	+‡	–	–	2/5
Total = 19			64/108 (59%)	25/64 (39%)	9/19 (47%)	6/19 (32%)	4/19 (21%)	17/62 (27%)

Data shown for 19 of 23 women with at least one evaluable specimen on ≥ 2 visits.

*HIV-1-specific lysis defined by effector CTL recognition and lysis of autologous target cells expressing either HIV-1 Env, Gag, or Pol at an E/T of 1:1 to 5:1. NK lysis defined by cytotoxicity of K562 target cells at an E/T of 4:1.

‡HIV-1-specific CTL clones generated.

§ND, not done.

gene (22), cultured in R-10 supplemented with 1.5 mg of G418 (neomycin) per ml, and selected by panning onto CD4 mAb-coated MicroCollector flasks (Applied Immune Sciences, Santa Clara, CA). Two million CD4-expressing LCL (B-LCL-CD4) were infected in 24-well plates for 7 d with 200–400 TCID₅₀ of HIV-1_{LAI} or HIV-1 from the supernatant of a PBMC co-culture (one passage) derived from patients with early HIV-1 infection (no. 1002 and no. 1005), according to the previously described method (22). HIV-1 infectivity of the B-LCL-CD4 was determined by reverse transcriptase assay, as previously described (23). Uninfected B-LCL-CD4 were used as controls for the HIV-1-infected targets. The erythroleukemia cell line K562 (ATCC CCL 243), maintained in R-10, was used to measure NK cell lytic activity. Uninfected and HIV-1 infected B-LCL-CD4, B-LCL infected with various rVV, and K562 cells were labeled with 0.1 mCi ⁵¹Na₂CrO₄ (NEN Products, Boston, MA) for 16 h. Thereafter, cells were gently washed three times and resuspended in R-10 at a concentration of 5×10^4 /ml.

CTL Chromium Release Assay. Radiolabeled target cells were

plated at 5×10^3 cells/well in 96-well round-bottomed plates. Effector cells were added in triplicate to the wells at varying effector to target cell ratios (E/T) in a total volume of 200 μ l. After a 5-h incubation at 37°C and 5% CO₂, 30 μ l of supernatant were harvested from each well into Lumaplates (Packard, Meriden, CT) and the ⁵¹Cr release was measured using a Topcount (Packard, Meriden, CT). In all experiments, spontaneous ⁵¹Cr release, measured from control wells containing target cells with media alone, was less than 25% of maximum lysis of target cells with 5% Triton-X (Sigma). The percent specific lysis was calculated as follows: $100 \times (\text{test release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$. A positive response was defined as (10% lysis of the target cells expressing HIV-1 gene products minus the appropriate control target cells).

Generation and Analysis of CTL Clones. Wells containing cells with HIV-1-specific cytolytic activity were plated at 1–3 cells per well in round-bottomed 96-well plates (Costar, Cambridge, MA) with 10^5 freshly isolated allogeneic γ -irradiated PBMC feeder cells, 25 U/ml rIL-2 and 30 ng/ml OKT3 in a total volume of

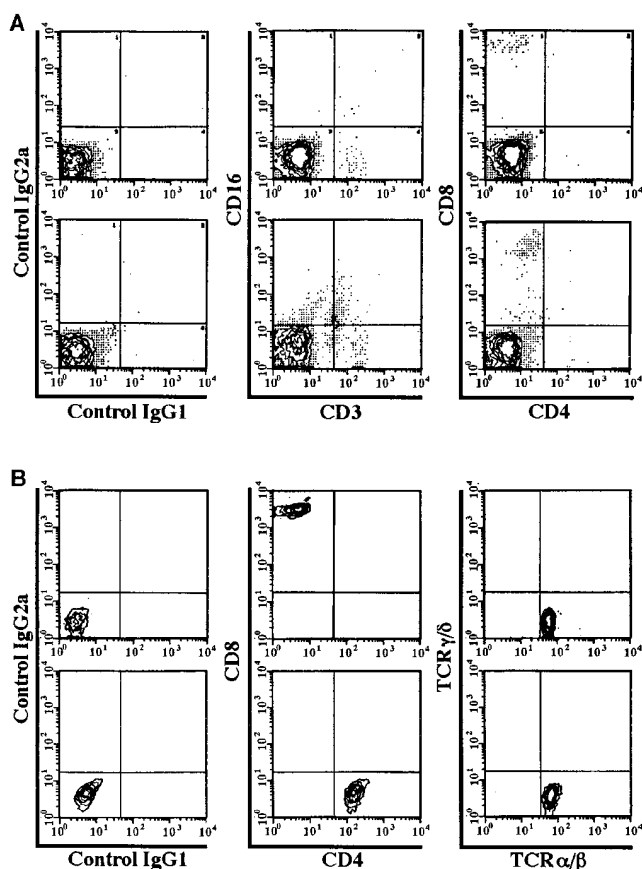


Figure 1. T lymphocytes in fresh cervical preparations and following clonal expansion. Two-color cytofluorimetric analysis of freshly isolated cervical mononuclear cells (A) and cervical CTL clones (B) obtained from HIV-1-infected women: volunteer 737 (A, upper panels), volunteer 747 (A, lower panels), and volunteer 710 (B, both panels). Left-hand plots represent the two antibody isotype controls (IgG1 FITC and IgG2a PE). (A) Middle plots demonstrate expression of CD3 (FL1) and CD16 (FL2). Surface expression of CD4 (FL1) and CD8 (FL2) are shown in A (right-hand plots) and B (middle plots). TCR $\alpha\beta$ (FL1) and TCR $\gamma\delta$ (FL2) are shown in B (right-hand plot).

200 μ l with R10-HS. HIV-specific lytic clones were expanded over 2–4 wk into 24-well culture plates. Epitope mapping and specific HLA restriction analysis was performed using autologous or HLA-mismatched allogeneic B-LCL infected with rVV or pulsed with the appropriate peptide.

Statistical Analysis. Statistical analyses were performed using Epi-Info6 software. The student *t* test was used to analyze normally distributed variables and the Mann-Whitney test for non-parametric analyses when a high coefficient of variation was observed. A type I error rate for each test was set at 5%.

Results

Cervical T Cells: Isolation and Phenotype

23 HIV-infected women enrolled in the study and provided at least two cytobrush specimens (Table 1). The study group overall was clinically stable, with a mean CD4⁺ T cell count of 470 cells/ μ l (median 450 cells/ μ l). From the evaluable cervical cytobrush specimens, 10,000–

2,500,000 mononuclear cells were isolated. No mononuclear cells were detected in four patients, one of whose physical deformity rendered pelvic examination difficult. Eight subjects presented with vaginal candidiasis and two with cervical dysplasia during the study. These events had no impact on the ability to isolate mononuclear cells from the cytobrushes, but in the case of vaginal candidiasis, the growth of T cells in culture was impaired.

By cytofluorimetric analysis of fresh, filtered cytobrush specimens, CD3⁺ T cells commonly comprised \leq 5% of the mononuclear cell population (Fig. 1 A) but could proliferate upon antigen-specific stimulation to purified CD4⁺ or CD8⁺ T cell populations (Fig. 1 B). Of these, typically $<$ 2% were CD4⁺ or CD8⁺ T cells, as shown in Fig. 1 A in two representative donors. The CD3⁻CD16⁺ NK cells were present in variable frequencies, ranging from 0–71% (median 23%) of the five donors examined. Because the T cells were few in the initial specimen, the phenotype of the T cells isolated on the day of collection did not predict the ability to later demonstrate CD8⁺ or CD4⁺ cytolytic activity in cell lines or clones.

We observed growth in wells containing cervical mononuclear cells after 1–2 wk in culture in 19 of the 23 donors, and in 64 of 108 (59%) specimens in the 19 donors over a mean of 5.7 (median 5.5) visits (Table 1). Of the 57 specimens that were not evaluable among the 23 women, $<$ 10,000 mononuclear cells were enumerated after the density centrifugation in 23 (40%), no growth was observed in wells upon antigen stimulation in 26 (46%), and contamination with yeast was noted within 2–5 d after culture in 8 (14%).

Cytolytic Responses

Although freshly activated HIV-1-specific CTL can be found in peripheral blood of seropositive individuals, with so few T cells present in the cervical specimens we opted to test only antigen-stimulated cervical T cells for their ability to recognize and lyse autologous target cells expressing HIV-1 gene products. Experiments in 6 donors are represented in Fig. 2 and in the aforementioned 19 donors are summarized in Table 1. HIV-specific cytolytic T cells were detected at E/T of 1:1 to 5:1 in nearly two-thirds (12/19, 63%) of patients with at least one evaluable specimen, and in over one-third (25/64, 39%) of the evaluable specimens (Table 1). In decreasing order of frequency, lytic activity was directed against autologous target cells expressing HIV-1 Env (9/19, 47%), Gag (6/19, 32%), and Pol (4/19, 21%). In five patients, CTL responses were detected against more than one gene product. In addition to HIV-1-specific lytic activity, 17/62 cervical specimens (10 of the 19 donors) contained NK cytolytic responses against the target cell line, K562, with specific lysis ranging from 0–71% at an E/T of 4:1. By contrast we tested similarly 5 HIV-1-uninfected women for the presence of cervical HIV-1 CTL, and lysis of autologous B-LCL infected with rVV (vPE-16, vDK-1, or vRT) by the antigen-stimulated cell lines was $<$ 5%. These results indicated that the *in vitro* stimulation method is unlikely to prime *de novo* cytolytic responses.

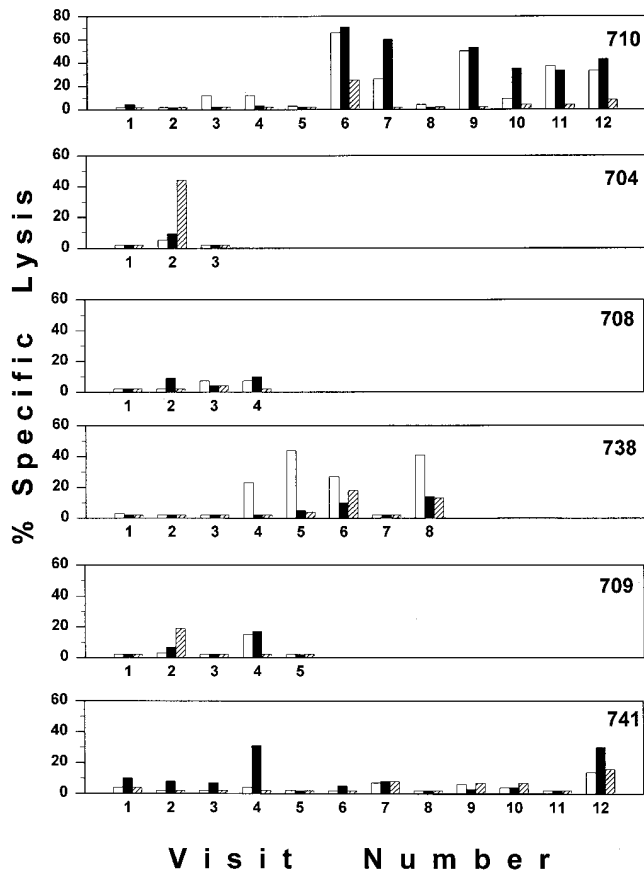


Figure 2. Cervical CTL are detected intermittently in HIV-infected females. The graphs depict responses in six HIV-1-infected adult females (donor number at upper right) tested at 4–8 weekly intervals. Percent specific lysis directed against autologous B-LCL expressing either Env (vPE-16) (open bar), Gag (vDK-1) (black bar), or Pol (vRT) (lined bar) is represented at an E/T ranging from 1:1 to 5:1. Lysis of the control target cells infected with vaccinia alone was <5% in each experiment and is not shown.

To explore factors associated with the presence of HIV-specific CTL, we stratified by the proportion of evaluable specimens and the mean CD4⁺ count of the subjects during the study period. Volunteers in whom at least 50% of cervical specimens contained sufficient cells for *in vitro* stimulation were no more likely to exhibit cytotoxic responses ($P = 0.16$) or have high (≥ 500 cells/ μl) CD4⁺ T cell responses ($P = 0.6$). The ability to detect HIV-1-specific CTL was significantly greater in patients with CD4⁺ counts ≥ 500 cells/ μl than in those with < 500 cells/ μl (8/8 vs 4/11, respectively, $P = 0.008$) (Table 1). Moreover, the presence of HIV-1 gag-specific CTL was strongly associated with a higher CD4⁺ count ($P = 0.006$). The median CD4⁺ count was 698 cell/ μl in those subjects with a Gag-specific CTL in comparison to a median of 356 cells/ μl in those lacking a Gag-specific response. These results imply that women with relatively intact immunity may be more likely to mount a mucosal immune response to HIV infection.

To examine the persistence of specific CTL in the cervix, we sampled the infected volunteers at monthly 4–8-wk in-

tervals. HIV-1-specific cytolytic responses directed against the three HIV-1 structural gene products were intermittent in most subjects when tested after one cycle of *in vitro* stimulation at low E/T (5:1 to 10:1) (Fig. 2 and Table 1). Some donors such as 710 and 738 demonstrated frequent CTL to more than one gene product (Fig. 2). An HIV-1 Gag-specific CTL response predominated in donor 741, while a Pol-specific response was substituted by an Env- and Gag-specific response in Donor 709. Upon review of the history and physical findings recorded at each visit during which specimens were collected, there were no obvious changes in clinical disease or cervical inflammation that accounted for the variability in CTL responses. In addition, positive CTL responses were detected repeatedly in some volunteers (no. 710, 738, 709, and 741 in Fig. 2) during various parts of the menstrual cycle (midfollicular to luteal phase) except during menstruation, suggesting that detection of such responses was not influenced by the reproductive cycle.

Comparison of Cervical T Cell Populations Obtained by Biopsy and Cytobrush

To compare the cell population extracted from a cervical cytobrush and an endocervical biopsy, simultaneous cervical cytobrush and biopsy specimens were obtained from two patients. In the case of the biopsies, the tissue was extensively washed before separation of the mononuclear cells to prevent contamination by blood cells. The mononuclear cell yields from the cervical biopsies were low ($< 10,000$), which did not permit cytofluorometric analysis on the initial analysis. After antigen-specific stimulation, a comparison of mononuclear cell phenotypes was performed in one donor, and cytolytic responses in both donors. In donor 775, the mononuclear cells expanded from the brush and biopsy after a 14-d stimulation were analyzed phenotypically by flow cytometry, which demonstrated 95 and 98% of the cells as CD4⁺, < 1 and 3% as CD8⁺, 99 and 98% as TCR α/β ⁺, < 1 and $< 1\%$ as TCR γ/δ ⁺, and 20 and 11% as MLA-1⁺, respectively. In both the cytobrush and biopsy specimens from the two donors, low level Env-specific lysis was detected at E/T 5:1 in patient 775, 10 and 16%; and in patient 708, 18 and 16%, respectively. These results indicate a relative concordance in the cell phenotype and cytolytic responses in the cervical cytobrush and biopsy specimens.

Analysis of Cervical CTL Clones: Phenotype, MHC Restriction, and Epitope Specificity

To determine the phenotype, MHC restriction, and epitope specificity of the mucosal CTL, we generated clones from cell lines that demonstrated HIV-specific lytic activity. As indicated in Table 1, Env-specific clones were established from four donors, Gag-specific clones from three donors, and Pol-specific clones from three donors. By flow cytometry (Fig. 1 B), the cell lines or clones examined were CD3⁺ (median 95%), TCR $\alpha\beta$ ⁺ (median 96%), TCR $\gamma\delta$ ⁻ (median 1%), CD16⁻ (median 1%), and MLA-1⁻ (median

Table 2. CD4⁺ CTL Clones from the Cervix Recognize HIV-1 Env gp41

	% Specific Lysis of Autologous Targets Infected with rVV*					
	vPE16 0-851aa	vPE17 0-747aa	vPE18 0-635aa	vPE8 0-502aa	vPE21 0-287aa	vPE22 0-204aa
Donor 738 [‡]						
C7	18	24	2	5	<1	<1
C9	23	40	5	6	8	5
C17	24	47	2	6	3	3
C18	11	43	6	6	7	2
C24	20	45	1	5	5	3
Donor 708 [‡]						
C10	18	13	<1	<1	<1	ND
C18	19	15	<1	<1	<1	ND

*Tested at an E/T of 5:1 against autologous B-LCL infected with recombinant vaccinia expressing serial truncations of HIV-1 envelope. Lysis of the vaccinia control targets ranged from <1-3%.

[‡]Clones were generated from cervical cytobrushes obtained at visit 4 (Figure 2) for each donor.

4%). All clones with specificity for HIV-1 Env were phenotypically CD4⁺ cells, and those with specificity for either HIV-1 Gag or Pol were CD8⁺ cells.

CD4⁺ Cervical Lymphocytes Mediate HIV-1 Env-Specific Lysis. Clonal expansion of Env-specific CTL was performed in four donors. In all cases, the clones generated were CD4⁺ by flow cytometric analysis (see Fig. 1 B for example in donor 710). To determine the envelope region containing the CTL epitope, the CTL clones in two donors were tested for lysis of autologous B-LCL infected with recombinant vaccinia containing serial truncations of the *env* gene (Table 2). Five clones from donor 738 and two clones from 708 recognized and lysed autologous targets infected with vPE16 and vPE17 but not vPE18, vPE8, vPE21, and vPE22 (Table 2). These results indicate that the CTL epitope recognized by both donors was within the gp41 region of HIV-1 envelope.

To assess the ability of the gp41-specific CTL clones to recognize and lyse HIV-1-infected targets, the two clones from donor 708 were tested for cytolysis of CD4-transduced B-LCL infected with either HIV-1_{LAI} or one of two primary low passage HIV-1 isolates, 1002 or 1005, or the uninfected control target. The clones lysed autologous targets infected with all three HIV-1 isolates but not the uninfected control targets (Fig. 3). The clones also lysed the partially mismatched targets (donor 738) but not the completely mismatched targets (donors RML and SCHU), indicating MHC class II restriction to either DR1 or DQ1 (Fig. 3). Additional studies to specifically define the class II haplotype were not performed. These studies indicate that CD4⁺ CTL are present in the cervix of HIV-infected women which are capable of killing HIV-1-infected cells.

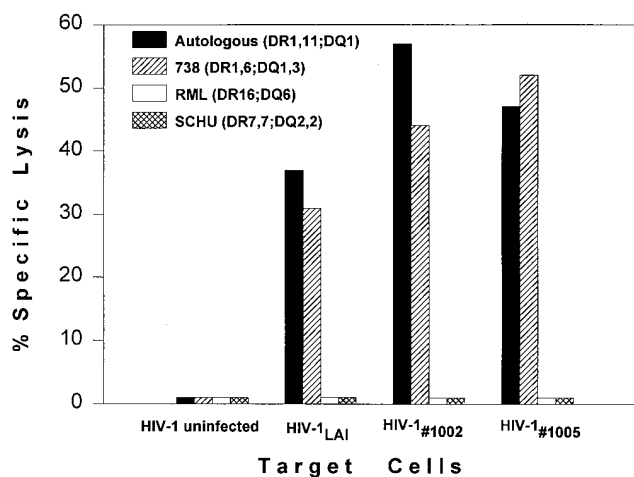


Figure 3. CD4⁺ cervical CTL clones from subject 708 lyse HIV-1-infected cells (E/T of 5:1). HIV-1 Env-specific clones lyse HIV-1-infected autologous and MHC class II partially mismatched (donor 738) B-LCL but not MHC class II completely mismatched (donors RML and SCHU) B-LCL or HIV-1-uninfected cells. The CTL clones recognize epitopes expressed by the laboratory-adapted strain, HIV-1_{LAI}, and two primary HIV-1 isolates, no. 1002 and no. 1005.

CD8⁺ Cytolytic Cells from the Cervix. In donor 738, four CD8⁺ clones were derived from the cervical cell line at visit 8 (Fig. 2). These clones recognized and lysed targets expressing HIV-1 Pol with a specific lysis ranging from 41-61% at an E/T of 3:1 (Fig. 4 A). To determine the MHC restriction patterns of these CTL clones, cytolysis of vRT-infected B-LCL partially matched at MHC class I loci was compared to vRT-infected autologous B-LCL. As shown in Fig. 4 A, lysis of the Pol-specific target cells was restricted to HLA-A2, with comparable lysis of targets from donors 115 (allo-1) and 5224 (allo-2) who share this haplotype. To identify the epitope recognized by the MHC-restricted CTL clones, we tested two nonamer peptides previously reported to be associated with HLA-A2-restricted HIV-1 Pol CTL (24). The 4 clones lysed autologous targets pulsed with the synthetic peptide spanning aa 476-484 but not the peptide spanning aa 346-354 of the Pol region (Fig. 4 B). To ascertain the ability of these CTL clones to lyse not only target cells expressing Pol through infection with recombinant vaccinia or pulsing with peptides, but also infected with HIV-1, we tested 4 clones against autologous B-LCL targets infected with either HIV-1_{LAI} or one of two primary HIV-1 isolates obtained from PBMC co-cultures of recently infected patients. As shown in Fig. 4 C, lysis by the four clones was observed at an E/T of 3:1 against targets infected with the laboratory-adapted strain HIV-1_{LAI} (49, 38, 39, and 22%), but not against targets infected with the two primary isolates (specific lysis <1%) or against the control uninfected targets (lysis ≤1%). In summary, these experiments demonstrate that the HLA-A2-restricted cervical CD8⁺CTL clones derived from this subject recognize a known epitope within HIV-1 Pol characteristic of peripheral blood CTL, and that the mucosal CD8⁺ CTL, in

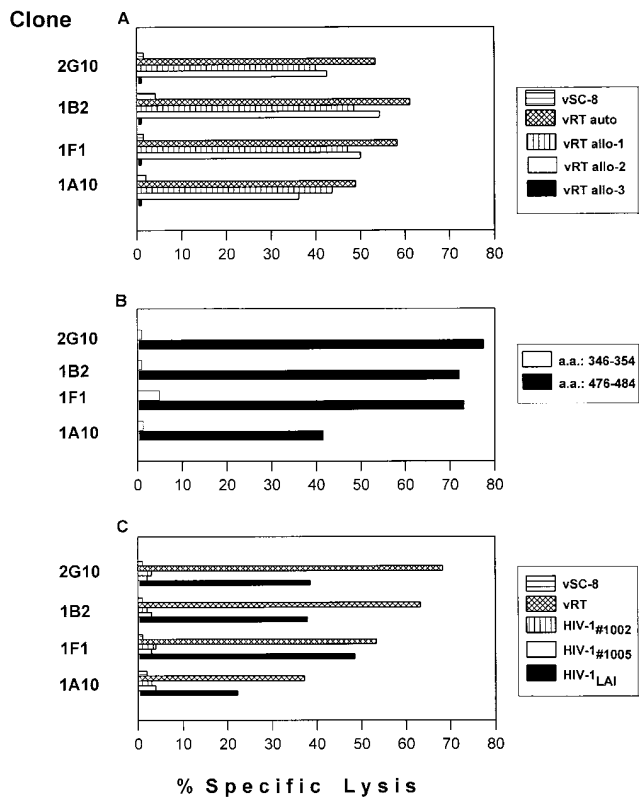


Figure 4. Cervical CTL clones derived from subject 738 (visit 8) are restricted by HLA-A2 and recognize targets expressing HIV-1 Pol aa 476-484 or HIV-1-infected cells. (A) Specific lysis of autologous and MHC class I partially matched allogeneic B-LCL infected with either vRT or the control, vSC-8, at an E/T 3:1. Serological HLA class I typing for the donors are the following: autologous, (A2,74, B58,63, Bw4,w6); allo-1, (A2,28,B44,14,Bw4,w6); allo-2, (A2,B27,44,Bw4); allo-3, (A23,74,B70,57, Bw4,w6). (B) Lysis by clones of autologous B-LCL pulsed with synthetic peptides spanning Pol amino acid region 346-354 (VIYQYMDDL) and 476-484 (ILKEPVHGV) at an E/T of 3:1. C. Lysis at E/T of 3:1 of HIV-1-infected and uninfected autologous B-LCL. Targets were infected with either HIV-1_{LAI} or one of two primary patient isolates, no. 1002 or no. 1005.

contrast to the mucosal CD4⁺ CTL, may function to destroy some but not all HIV-1 strains.

Patient 710 had a vigorous Env- and Gag-specific CTL response during the majority of monthly visits (Fig. 2). To determine the breadth of CD8⁺ CTL epitope specificity, we chose to clone the Gag-specific cervical CTL lines from visits 8 and 11. Three CD8⁺ CTL clones derived from the cytobrush specimen taken on visit 8 (Fig. 1 B) and six clones from visit 11 demonstrated lytic activity to Gag-expressing autologous B-LCL (Table 3). As shown in Fig. 5, A and B, lytic activity of these CD8⁺ clones was restricted by either class I HLA-B14 or HLA-B27. All cervical-derived clones recognized targets expressing Gag epitopes within the p24 region: the B14-restricted clones lysed targets pulsed with the 20-mer spanning aa 290-309, and the B27-restricted clones, the 20-mer spanning aa 160-179 (Table 3). Thus, the patterns of gag epitope recognition in the cervical mucosa were consistent in the two time periods separated by a four month interval.

Comparison of the CTL Repertoire in Cervix and Peripheral Blood. To compare the MHC restriction patterns and epitope specificities of the CTL derived from the cervical mucosa with that from peripheral blood, we also established gag-specific CTL clones from PBMC obtained from patient 710 at visit 8. Six CD8⁺ gag-specific CTL clones derived from the peripheral blood were restricted by the same class I HLA haplotypes, B14 and B27, as in the cervical clones derived from visit 8 and 11 (see Fig. 5 for representative experiments in four clones). Four blood clones were tested in more detail for epitope specificity. Two of the four shared the same epitope recognition patterns (Table 3): HLA-B14-restricted CTL clone 1A8 lysed p24 peptide 20 (aa 290-309), and HLA-B27-restricted clone 4B11 lysed peptide 7 (aa 160-179). However, two other peripheral blood clones demonstrated different epitope specificities: the B14-restricted 4F10 clone recognized the p24 peptide 5 (aa 140-159) and the B27-restricted 1H8 recognized an epitope within the p17 region, as evidenced by <1% lysis of the overlapping p24 20-mer peptides, and 43% lysis of the autologous target infected with vp1290 expressing Gag p17 at an E/T of 4:1. These results indicate that the MHC restriction patterns and epitope specificities of the mucosal CTL may be similar to those of the systemic CTL.

To address this issue in another donor, subject 909, we examined the CTL repertoire from the cervix and blood obtained on the same visit. HIV-1-specific CTL were detected recognizing HIV-1 Gag but not Env or Pol in both mucosal and systemic compartments. Eight CD8⁺ lytic clones were generated from the cervix and five from blood, all of which recognized HIV-1 gag p24 (Table 4). The cervical clones were restricted by HLA-B57, with specific lysis of the partially mismatched Gag-expressing targets sharing HLA-B57 ranging from 37-58% at E/T of 5:1. Of the 5 peripheral blood Gag-specific clones, only 3 (2B2, 4B10, and 4C7) were restricted by HLA-B57. All eight cervical clones and the three HLA-B57 peripheral blood clones (2B2, 4B10, and 4C7) recognized the Gag p24 20-mer spanning aa 160-79 (Table 4). PBL-derived clones 3B9 and 3A3 lysed vp1289 (specific lysis 75% and 74%, respectively), a recombinant vaccinia expressing Gag p24, but not vp1290 (specific lysis 5% and <1%, respectively) expressing Gag p17. Additional studies indicated recognition and lysis by clones 3B9 and 3A3 of a p24 gag peptide (LADP788.21) spanning aa 333-352 (26% and 16% specific lysis, respectively). The findings in donor 909 together with those in 710 above indicate that the CTL repertoire in the cervix is commonly found in peripheral blood of naturally infected women.

Discussion

This investigation provides the first evidence that HIV-1-specific cytotoxic T cells are present in the cervical mucosa of infected women. Cervical CTL were detected at least once in nearly two-thirds (63%) of the subjects tested and in approximately half (47%) of their specimens with repeated examinations (Table 1), which indicates that muco-

Table 3. Comparison of CTL Responses in the Cervix and Peripheral Blood in Donor 710*

CTL clone	% Specific Lysis*					
	Vac control	vDK (gag)	v1290 (p17)	p24 peptide 5 [§] aa140-159	p24 peptide 7 aa160-179	p24 peptide 20 [¶] aa290-309
Cervical-derived visit 8						
8D4 (HLA-B14) [‡]	0	25	ND	4	4	36
8B3 (HLA-B27)	0	44	ND	4	55	2
6G11 (HLA-B27)	0	33	ND	0	38	3
Cervical-derived visit 11						
1F2 (HLA-B14)	0	33	ND	0	0	54
1F6 (HLA-B14)	0	41	ND	0	0	61
1H12 (HLA-B14)	0	45	ND	0	0	49
4A10 (HLA-B14)	0	50	ND	0	0	57
3G7 (HLA-B27)	0	23	ND	0	37	0
4B12 (HLA-B27)	0	24	ND	0	43	0
Blood-derived, visit 8						
1A8 (HLA-B14)	0	45	0	5	4	51
4F10 (HLA-B14)	1	52	ND	60	3	0
4B11 (HLA-B27)	1	81	ND	3	30	ND
1H8 (HLA-B27)	0	55	43	0	0	0

*Specific lysis of autologous B-LCL infected with either vaccinia control, vDK expressing HIV-1_{LAI} Gag, or v1290 containing Gag p17, or pulsed with synthetic 20-mers spanning the p24 region. PBMC clones were tested at E/T of 4:1, cervical clones from visit 8 at E/T of 3:1, and cervical clones from visit 11 at E/T of 5:1.

[‡]Class I HLA restriction, see also Fig. 4.

[§]p24 Peptide 5, aa 140-159: GQMVHQAI SPR TLN AWVKVV.

^{||}p24 Peptide 7, aa 160-179: EEKAFSPEV I P M F S A L S E G A.

[¶]p24 Peptide 20, aa 290-309: PKEPFRDYVDRFYKTLRAEQAS.

sal cytolytic responses are common in asymptomatic HIV-infected individuals. Both CD4⁺ and CD8⁺ T cells mediate the MHC-restricted cytolytic activity, which can be directed to one or more epitopes within HIV-1 proteins encoded by *env*, *gag*, or *pol*, and can recognize and lyse HIV-1-infected cells. Thus, HIV-1 infection induces not only a profound CTL response in peripheral blood, but also in tissues reservoirs such as lymph nodes (Musey, manuscript in preparation) or other lymphoid organs (25), central nervous system (26), respiratory tract (27), and mucosal surfaces.

Sampling by the cytobrush method to investigate cellular immunity in the cervix offers the advantages of being well-tolerated, non-invasive, inexpensive and practical. Because the procedure occasionally can cause bleeding, a number of precautions were implemented in this study to avoid contamination of the cervical specimens with blood. These included collection from volunteers only at times when they were not menstruating or had no evidence of epithelial disruption, sampling for this study before other procedures, rotating the cytobrush gently only one complete turn, and rejecting samples with visible blood or microscopic RBC. Thus, we believe that our samples were mucosal in origin and not tainted with blood. The concordance of the find-

ings from the cytobrush specimens with those from the cervical biopsy with respect to cell phenotype and cytolytic function further support the mucosal derivation of these cells and is consistent with previous reports (8). Also, our findings in HIV-1-infected humans are similar to those previously observed by Lohman and colleagues (7), who reported SIV-specific cytotoxic T lymphocytes in the vaginal mucosa of infected monkeys.

The HIV-1-specific T cells in the cervical cytobrush specimens are likely derived from an intraepithelial lymphocyte population. These originate either directly from primary lymphoid tissue, similar to gastrointestinal intraepithelial cells which survey mucosal pathogens, or more likely from memory and effector lymphocytes generated from naive cells in secondary lymphoid organs which localize to epithelial sites upon antigenic stimulation. The effector T cells exhibited the TCR $\alpha\beta$ ⁺ rather than the TCR $\gamma\delta$ ⁺ phenotype (Fig. 1), which is consistent with CTL found in the cervicovaginal epithelium of SIV-infected macaques (7) and in some cases with intraepithelial gastrointestinal T cells (28). The low level of surface expression of the human mucosal lymphocyte antigen 1 (CD103), an alpha E beta 7 integrin, on mucosal T cells from both the cytobrush and

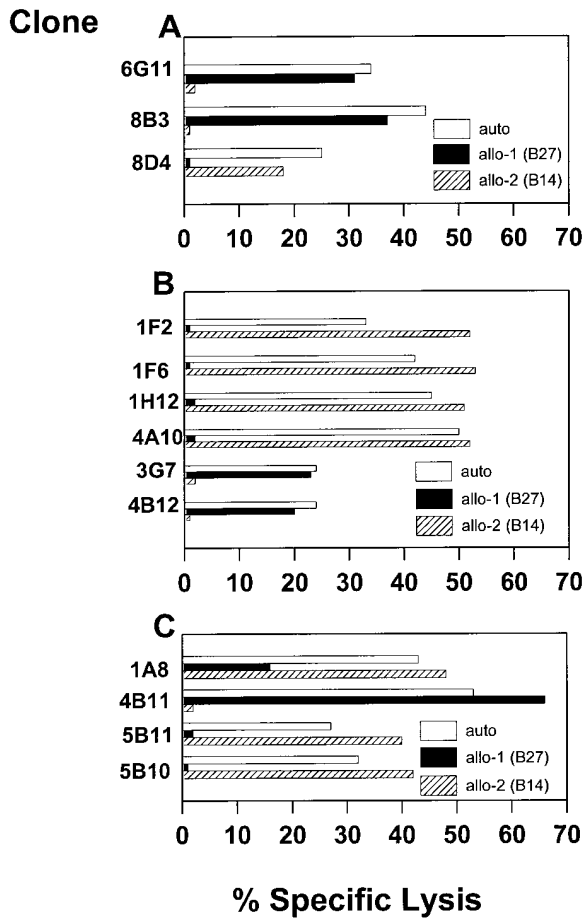


Figure 5. Gag-specific CTL clones from the cervix and blood of Subject 710 are HLA class I restricted. Gag-specific clones were tested for lysis of B-LCL infected with vDK-1 (Gag) or vSC-8 (control). Class I MHC-restricted lysis by the clones is evidenced by lysis of the Gag-expressing autologous B-LCL (A24,B14,B27) and B-LCL from donors sharing one class I HLA allele as indicated on the figure. Lysis of the control targets infected with vSC-8 (vac-lac) were <2% for all targets. (A) Three cervical CTL clones from visit 8, tested at an E/T of 3:1. (B) Six cervical CTL clones from visit 11, tested at an E/T of 5:1. (C) Four PBMC-derived CTL clones from visit 8, tested at an E/T of 4:1.

biopsies differs from previous findings on T cells from other mucosal sites such as the gut and lung (29–31). The disparate results suggest that MLA-1 may be either differentially regulated in the reproductive mucosa in contrast to gut mucosa or its surface expression down-regulated as a consequence of in vitro stimulation and differentiation into effectors.

Although our study was not designed to evaluate cofactors that may be associated with a CTL response in the cervix, there were clearly differences in the ability to detect CTL responses relative to CD4⁺ count. These findings suggest that the ability to mount a local immune response may in part require a more intact immune system. This is consistent with findings in other cervicovaginal infections such as HPV (14, 32) and in neoplasia (32), whereby cellular immunodeficiency enhances progression of the cervical disease. Moreover, one may anticipate that the failure to control vi-

Table 4. HIV-1 Gag-specific CD8⁺ CTL Clones Derived from the Cervix and Blood of Donor 909 Share Some but not All Epitope Specificities

Clone	% Specific Lysis*			
	Vac Control	vDK	p24 aa 150-169	p24 aa 160-179
Cervical-derived				
C4	0	47	0	60
C6	0	50	0	65
C12	0	41	0	52
C29	1	37	0	43
C35	0	58	0	76
C43	0	39	0	45
C48	0	41	0	52
C52	1	63	0	79
Blood-derived				
2B2	0	42	0	55
4B10	0	33	0	35
4C7	0	52	3	78
3A3	1	55	0	4
3B9	0	40	0	0

*Specific lysis at E/T of 5:1 of targets either infected with the vaccinia control or vDK expressing HIV-1 gag, or pulsed with p24 gag peptide 6, aa150-169 (RTLNAWVKVVEEKAFSPEVI) or peptide 7, aa160-179 (EEKAFSPEVPMFSALSEGA). Clones 3A3 and 3B9 lysed the p24 gag peptide spanning aa 333-352 (ILKALGPAATLEEMMT-ACQG) at 26 and 16%, respectively.

ral replication by cervical CTL renders the HIV-1-infected host more susceptible to local disease, such as HPV-associated cervical lesions and chronic vaginal candidiasis, which are well-known to contribute significant morbidity in women with declining CD4⁺ counts (14, 33–36). Our results also indicate that the presence of cervical cytolytic responses may be intermittent, and the factors which control their presence are not known. Potential influences currently under study include the presence of HIV-1 or other infections and host factors such as antigen presenting cell function, the cytokine milieu, expression of adhesion molecules, production of lymphocyte chemoattractants, and hormonal cycling.

We provide two comparisons of the CTL repertoire in the cervix and peripheral blood (Tables 3 and 4). These results suggest that the mucosal response may be narrower than the systemic response. However, there may have been sampling bias relative to the two compartments, and the cloning efficiency of the T cells from the cervix may be lower than from peripheral blood and thus the breadth of responses in the cervix may have been missed. Although it is impossible to completely discount this, the likelihood is low since typically the percentage of clones generated from

the initial T cell yields were greater in the cervix than from peripheral blood. Alternatively, the virus populations in the cervix may be distinct from those in blood, which may account for the differences in CTL repertoire in the two compartments. In support of this, recent investigations demonstrate heterogeneous viral populations in the cervix of infected women which are not always reflected by virus in PBMC (37). In addition, it has been postulated that HIV-1 infection like other infections may be associated with clonal expansion followed by clonal exhaustion coincident with an inability to clear virus (38), and these events may be occurring locally at the site of infection and perhaps distinct from peripheral blood. Studies are in progress to address these issues, particularly with regard to analysis of local viral quasispecies and TCR usage by the HIV-1-specific CTL clones, which should further elucidate the mechanism by which mucosal CTL may control the evolution of specific HIV-1 variants.

In addition to MHC-restricted HIV-1-specific T cells, we identified natural killer cell activity in the mucosal epithelium (Table 1). When females are exposed to HIV-1 by sexual contact, the first line of defense against HIV in the genital mucosa likely are nonspecific effector mechanisms such as NK cell activity. Our findings support this hypothesis, with a variable number of NK cells found in the fresh specimens and the majority of subjects containing NK cy-

tolytic activity upon in vitro culture. This may be followed by the generation of antigen-specific immune responses, such as specific antibody, IgA or IgG, which mediate attachment, opsonization, neutralization or antibody-dependent cellular cytotoxicity, as well as cytotoxic T lymphocytes. Infected cells are likely to first encounter CTL effector responses in the draining lymph nodes, but the continued presence of HIV-1-specific CTL may reflect the persistence of antigen in the cervical mucosa.

What remains to be determined is the role these effector cells play in controlling local HIV-1 infection and prevention of viral transmission. Because these cells are capable of destroying HIV-infected cells, their presence in the genital area may serve to control viral replication and thus prevent transmission to sexual partners. Moreover, these T cell responses can conceivably provide an important defense against HIV-1 exposure and thus may be useful to elicit with vaccines. Immunization strategies that target mucosal sites may prove more effective if designed to elicit both antibody and cellular responses locally. Thus, this investigation provides new evidence that T cell responses in the genital mucosa may be critical in the immune defense against HIV-1, and future studies to elucidate their role in protecting against transmission and controlling early infection should be further explored.

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